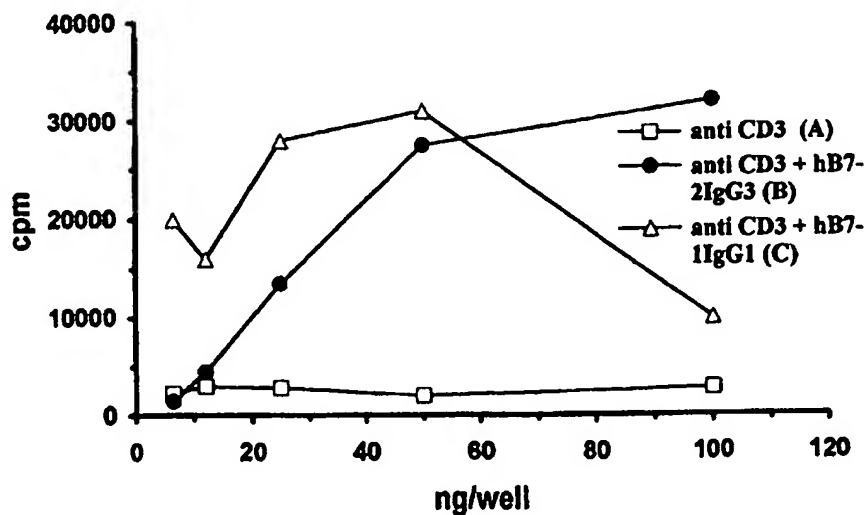




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(54) Title: METHOD FOR THE PRODUCTION OF ACTIVATED MARKED TUMOR-SPECIFIC T CELLS AND USE THEREOF IN TREATMENT OF TUMORS



## (57) Abstract

A method for the production of activated tumor-specific T cells by co-cultivating, ex vivo, tumor cells from a patient with T cells from that patient, comprising the steps of: i) incubating the tumor cells with a first fusion protein obtained from a B7 protein and one partner of a biological binding pair and a second fusion protein obtained from an antibody against a cell surface antigen and the other partner of the biological binding pair; ii) inhibiting the proliferation of the tumor cells prior to or after that incubation; iii) co-cultivating the tumor cells with the T cells to be activated, until activation of the T cells is attained; iv) separating the activated T cells from the tumor cells, is highly efficient and can be carried out in a simple manner.

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**Method for the production of activated marked tumor-specific T cells  
and use thereof in treatment of tumors**

The invention is concerned with a method for the production of activated marked tumor-specific T cells by co-cultivating tumor cells from a patient with T cells from that patient, a therapeutic composition containing such activated T cells as well as the use thereof in tumor therapy.

Tumor-specific T lymphocytes recognize peptides derived from proteins synthesized by tumor cells and presented on their cell surface by MHC molecules (Lurquin et al., Cell 58 (1989) 293 and Hellström, K.E., et al., The Biologic Therapy of Cancer, J.B. Lippincot Co., Philadelphia (1991) p.35). However, T cells require two activating signals to express full effector functions (Mueller, D.L., et al., Annu. Rev. Immunol. 7 (1989) 445). Signal 1 is generated when the T cell receptor (TCR) interacts with the MHC peptide complex. Signal 2 is provided by costimulatory molecules expressed by professional antigen-presenting cells (APC). Many tumors, particularly those of non-hematopoietic origin, do not express costimulatory molecules and thus fail to activate tumor-specific T lymphocytes (Chen, L., et al., Immunol. Today 14 (1993) 483). This finding has provided a rationale for the introduction of genes encoding costimulatory molecules into tumor cells to increase their immunogenicity and vaccination potential.

Among the different costimulatory molecules, B7 proteins (e.g. B7-1, B7-2 and B7-3) are of particular interest since they are expressed on professional APC (Vandenberghe, P., et al., Int. Immunol. 3 (1993) 229; Guinan, E.C., et al., Blood 84 (1994) 3261-3282; WO 95/03408). These costimulatory molecules interact with CD28 and CTLA4 counter-receptors expressed on most T cells leading to a marked increase of IL-2 production, proliferation and acquisition of effector function in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Azuma, M., et al., J. Immunol. 115 (1993) 2091). Blocking the ligation of B7 with a soluble CTLA4-Ig chimeric molecule provokes unresponsiveness in vitro, which has dramatic suppressive effects on the humoral response and graft rejection in vivo. In addition, it has been shown that the transfection of the B7-1 gene into different mouse tumor lines can lead, in some cases, to both their primary rejection and the establishment of a protective immunity (Chen, L., et al., J. Exp. Med. 179 (1994) 523 and Ramarathnam, L., et al., J. Exp. Med. 179 (1994) 1205). However, these

studies have revealed a limited efficiency of B7-1 activity on T cell-dependent tumor immunity.

The efficiency of B7 costimulation of anti-tumor T cells is enhanced by cooperation between B7 and ICAM-1, whereby an efficient tumor-specific immune response is stimulated. This effect is dependent on the recruitment of a potent inflammatory reaction (Cavallo, F., et al., *Eur. J. Immunol.* 25 (1995) 1154-1162).

Molecules of the B7 family are CD28 counter-receptors expressed on APCs. B7-1 was characterized and sequenced in Freeman, G.J., et al., *J. Immunol.* 143 (1989) 2714-2722. B7-2 and B7-3 were characterized and sequenced in Freeman, G.J., *Science* 262 (1993) 909-911 and WO 95/03408. The B7 molecules are members of the Ig supergene family with two Ig-like domains (IgV and IgC) and a transmembrane domain. It is suggested that the B7 molecules exist as a monomer or a homodimer on the cell surface, but little, if any, evidence suggests that it can form a heterodimer with CD28 (Lindsten, T., et al., *J. Immunol.* 151 (1993) 3489). The B7 molecules have a higher affinity for CTLA-4 than for CD28. The genes of the B7-1 and B7-2 molecules have been localized to chromosomal region 3q13.3-3q21. Though these molecules were not highly homologous at the DNA level, they share the identical Ig supergene family structure and the ability to bind to CD28 and CTLA-4, as mentioned above.

However, it was found that B7-1 and B7-2 differ in their appearance after B cell activation. B7-2 appears on the cell surface within 24 hours of B cell activation and B7-1 appears later (Boussiotis, V.A., et al., *Proc. Natl. Acad. Sci. USA* 19 (1993) 11059). It was further found that in unstimulated human monocytes B7-2 is constitutively expressed whereas B7-1 expression is induced after activation (Azuma, M., et al., *Nature* 366 (1993) 76). B7-3 is also described in Boussiotis et al. B7-3 has not yet been molecularly cloned.

In WO 95/03408 it is suggested to modify a tumor cell to express B7-2 and/or B7-3 by a transfection of the tumor cell with the nucleic acid encoding B7 in a form suitable for expression of B7 on the tumor cell surface. Alternatively, the tumor cell is modified by contact with an agent which induces or increases the expression of B7 on the tumor cell surface. It is further suggested to couple B7-2 and/or B7-3 to the surface of the tumor cell to produce a modified tumor cell. The term "coupling" as used in WO 95/03408 refers to a chemical, enzymatic or other means (e.g. antibody) by which B7-2 and/or B7-3 is linked to a tumor cell such that the costimulatory molecule (B7) is present on the surface of the tumor cell and is

capable of triggering a costimulatory signal in T cells. It is further suggested to cross-link B7 chemically to the tumor surface, using commercially available cross-linking reagents. Another approach would be to couple B7-2 and/or B7-3 to a tumor cell by a B7-specific antibody which binds to both the costimulatory molecule B7 and a cell surface molecule on the tumor cell.

The production of activated tumor-specific T cells may be accomplished by co-cultivating tumor cells from a patient, which tumor cells carry, on their surface, such a costimulatory molecule, with T cells from that patient. Modifying such tumor cells with B7 according to the known method involves a number of drawbacks, however, and is rather unsuitable for routine therapy. Transfecting the tumor cells with the nucleic acid encoding a costimulatory molecule usually is not very effective. In addition to this, it is necessary that the transfected and non-transfected cells should be separated, in a laborious procedure, prior to co-cultivation with the activated T cells. McHugh, R.S., et al., Proc. Natl. Acad. Sci. USA 92 (1995) 8059-8063 suggest to introduce B7-1 onto the surface of tumor cells by using a purified GPI (glycosylphosphatidyl-inositol) anchored B7-1 molecule (GPI-B-7) which is able to bind its cognate ligand CD28 and incorporate itself into tumor cell membranes after a short incubation. However, the stability of the GPI-B-7 on the surfaces of irradiated tumor cells is limited and the cells do retain only minimal presentation of B7 capable of effective binding to CD28.

Coupling of B7 to a tumor cell by using a B7-specific antibody which binds both the costimulatory molecule and the cell surface molecule of the tumor has also severe disadvantages. B7 antibodies which are described in the state of the art bind to B7 unfortunately in such a way that the binding of B7 to CD28 decreases dramatically or is completely inhibited. The reason for this is that all known anti-B7-1 and anti-B7-2 monoclonal antibodies interact with CD28 and thus inhibit the T cell response (Azuma, M., et al., J. Exp. Med. 175 (1992) 353-360; Azuma, M., et al., J. Immunol. 149 (1992) 1115; Azuma, M., et al., J. Exp. Med. 177 (1993) 845; Caux, C., et al., J. Exp. Med. 180 (1995) 1841-1847).

It is therefore the object of the present invention to provide a method for the production of activated tumor-specific T cells which can be carried out in a simple manner and exhibits a high efficacy.

The subject-matter of the invention is a method for the production of activated tumor-specific T cells by co-cultivating, ex vivo, tumor cells from a patient with T cells from that patient, comprising the steps of

- i) incubating the tumor cells with a first fusion protein obtained from a B7 protein and one partner of a biological binding pair and a second fusion protein obtained from an antibody against a cell surface antigen and the other partner of the biological binding pair;
- ii) inhibiting the proliferation of the tumor cells prior to or after that incubation;
- iii) co-cultivating the tumor cells with the T cells to be activated, until activation of the T cells is attained;
- iv) separating the activated T cells from the tumor cells.

T cells of a patient are isolated from peripheral blood lymphocytes (PBMC), which have been prepared from buffy coat of normal human blood samples (Dellabona, P., et al., J. Exp. Med. 177 (1993) 1763-1771). After centrifugation, the mononucleic cells are collected and propagated (Dellabona, P., et al., J. Exp. Med. 177 (1993) 1763-1771). From this preparation, CD4<sup>+</sup> and/or CD8<sup>+</sup> lymphocytes can be isolated by means of magnetic activated cell sorting (MACS).

The T cells which are used for activation can be generated from a patient according to known methods, preferably by a simple passage of PBMC on a nylon wool column, whereby B cells and monocytes are excluded (Julius, M.H., et al., Eur. J. Immunol. 3 (1973) 645). CD8<sup>+</sup> (with or without CD4<sup>+</sup>) T cells are purified from the peripheral blood of the patient in vitro by a sorting method, preferably by immunomagnetic sorting. In addition, it is preferred to use a mixed population of tumor-infiltrating T cells (TIL) and purified CD8<sup>+</sup> T cells which are obtained from a surgical tumor specimen according to Anichini, A., et al., J. Exp. Med. 177 (1993) 989.

The phrase "activated tumor-specific T cells" preferably denotes tumor-specific T cells which are capable of killing in a specific and restricted manner the tumor cells originally used to activate them. The activation is MHC restricted in the sense of Townsend, A., and Bodmer, H., Ann. Rev. Immunol. 7 (1989) 601.

Generation of tumor specific T cells: either total PBMC, or purified CD8<sup>+</sup> T cells are cultured in 24-well plates at a ratio of 10:1 to 5:1 with non-replicating tumor cells (irradiated or mit. C treated or both) which are either autologous or semi-allogeneic, in 2 ml of standard RPMI medium containing 5% human serum, at 37°C. Multiple cultures containing 2 to 5 millions of PBMCs or 1 to 2 millions of CD8<sup>+</sup> T cells can be set. The tumor cells have been prepulsed with a saturating concentration (to be determined depending on the different kind of

constructs) of soluble B7-1 or B7-2 Ig x antitumor mAb. Recombinant human IL-2 is added to the culture at 5 U/ml at day +5 of the culture and maintained until day +10, after which its concentration is raised to 10 U/ml. At day +15 of the culture, living T cells are recovered from the cultures by a centrifugation over a Ficoll gradient and re-stimulated using the same non-replicating tumor cells prepulsed with the recombinant B7-1 or B7-2 Ig x anti-tumor mAb at a ratio T cell/tumor of 2:1, and a concentration of one million T cells per ml. This restimulation step is performed in 24-well plates, in standard RPMI medium containing human serum supplemented with 2 U/ml recombinant human IL-2. At day +5, the concentration of rhIL-2 is raised to 10 U/ml and maintained as such until day +15.

The T cells can be restimulated a third time as described above, before being tested in a conventional cytotoxicity test (Lanzavecchia, A., Nature 319 (1986) 765-767) against the tumor cells used for re-stimulation in vitro, and unrelated tumor cells for control. The specificity of the T cell line for the tumor is judged according to the level of cytotoxicity shown in the assay. A specific killing activity of about 30-40% can be considered relevant for therapeutic interest. In this case, the tumor specific polyclonal T cell line can be expanded further using a polyclonal activator: PHA in the presence of irradiated allogeneic feeder cells (allogeneic PBMC) and 10 U/ml of rhIL-2; or anti-CD3 mAb + B7-1 IgM and 10 U/ml rhIL-2. At day +15 of restimulation, rhIL-2 is raised to 20 U/ml for 5 days and then to 50 U/ml for another 5 days. By different cycles of restimulation it is possible to reach the desired number of activated T cells to be reinfused in the patient.

In a preferred embodiment, the proliferation of the tumor cells is inhibited prior to or after the incubation according to step i). This may be accomplished, for instance, by means of irradiation or by use of mitomycin C. For irradiation, preferably 3000-5000 Rad are used for inhibition with mitomycin C, preferably 50-100 µg/million of cells are used. Mitomycin C is preferred for inhibition because it prolongs the survival of tumor cells during re-stimulation of T cells.

In a further preferred embodiment of the invention, the activated T cells are marked, preferably after activation. Such a marker preferably is a molecule which is presented on the surface of the marked cell. It is therefore particularly preferred to transform the T cells with the nucleic acid which codes for a protein which is presented on the surface of said cell. Such cell surface proteins or antigens are, for example, CD24 (J. Cell. Biochemistry Supplement 17E, page 203, abstract S210), LDL or NGF receptor (WO 95/06723).

After autologous transplantation of said activated T cells which are marked with such a gene product it is possible to trace these cells directly after transplantation in the patient. This gene marking will allow to monitor and compare the efficiency of the therapy with activated tumor-specific T cells.

In a preferred embodiment of the invention, the T cells can further be transformed with a suicide gene. Such a gene causes directly or by mediators the death of the infected cell (cf. WO 92/08796 and PCT/EP94/01573) for in vivo-specific elimination of these cells after successful therapeutic treatment. For this purpose, there is preferably applied the thymidine kinase gene, which confers to the transduced activated T cells in vivo sensitivity to the drug Gancyclovir for in vivo-specific elimination of cells. If, for example, the patient develops signs of an acute incompatibility of the activated T cells, for example like a graft versus host disease (GVHD), with increasing liver function enzymes and a positive skin biopsy, it is preferred to administer i.v. two doses of about 10 mg/kg of the drug Gancyclovir. This results in a reduction of marked activated T cells without a considerable reduction of other lymphocytes.

The diphtheria toxin gene is also preferred as a suicide gene, which is described in WO 92/05262. For the in vivo-specific elimination of the activated T cells, it is also possible to induce a cell apoptosis. It is thus preferred to use a modified FAS receptor and a dose of a related ligand.

The tumor cells of the patient are taken from a surgical specimen. An aliquot of tumor cells can be used to regenerate a tumor cell line either in vitro or in vivo in immunodeficient mice, for subsequent stimulation of T cells. The rest of the fresh tumor cells are used for the direct stimulation of the T cells. Such tumor cells are, for example, melanoma, carcinoma (e.g. breast, cervix, head and neck, colon, lung, kidney, stomach), sarcoma, lymphoma or leukemia.

The term "biological binding pair" is understood to mean a combination of two molecules which have a high specific binding capacity with respect to one another. Such binding pairs are, for example, biotin/avidin (or streptavidin/neutravidin), or sugar/concanavalin A. Preferably, the affinity constant of the binding is  $k_d < 1 \text{ nmol/l}$ . Preferably, higher affinity constants are applied. For this reason, the biotin/avidin or streptavidin interaction is preferred because of their high affinity. This interaction is stronger than any other receptor ligand in a body. Therefore, it can be used in vivo to conjugate two components of a bifunctional reagent. Methods of biotinylation are described in Harlow, E., and Lane, D., Antibodies, Cold Spring



Harbor Laboratory (1988) 341. Biotinylation or cross-linking with avidin or streptavidin or neutravidin is carried out according to the methods well-known to one skilled in the art.

A preferred technique for pairing two protein molecules is chemical crosslinking, which forms a stable covalent bridge. Many bifunctional crosslinkers are commercially available. There is particularly preferred SPDP (N-succinimidyl-3-(2-pyridyldithio)propionate) crosslinker.

As B7 protein, part or all of B7, preferably of B7-1, B7-2 or B7-3 can be used. Preferably, there are used parts of B7 proteins which include at least the N-terminal variable domain. There are particularly preferred B7-1 or B7-2 proteins and derivatives as described in WO 95/03408, which is for this matter incorporated herein by reference. Since the binding of the B7 fusion protein to the cell surface is effected via the interaction between the biological binding pair and the binding of the anti-surface antigen-antibody, it is not necessary that the B7 molecule should still contain the transmembrane domain.

The second fusion protein contains an antibody against a cell surface antigen and the other partner of the biological binding pair. As antibodies, antibodies that are directed against a great number of cell surface antigens can be used. Those cell surface antigens need not necessarily be tumor cell-specific surface antigens. For this reason, it is preferred to use antibodies against surface antigens which occur to a large extent on cell surfaces, such as ERB B2 or the transferrin receptor. It is also possible, however, to use antibodies against suitable tumor-associated antigens like: CEA for colon carcinomas, lung carcinomas, mammary carcinomas; CD33 for myeloid leukemias; CD19/CD20 CALLA and CD38 for B cell leukemias, lymphomas, myelomas; Met for gastric carcinomas; OVCA (MOV-18) for ovarian carcinomas; or melanoma-specific antigens.

Preferably, co-cultivation of the activated tumor cells with the T cells is carried out in the presence of small doses of lymphokines (such as IL-2, IL-6, IL-7) which, in addition, are capable of stimulating T cells well. Several rounds of re-stimulation are preferred in order to expand to large numbers tumor-specific effector T cells. Three to four days after the last re-stimulation in vitro, the tumor effector T cells are reinoculated i.v. into the patient. The number of T cells that must be transferred into the patient is variable and can be found out according to established protocols. Preferably, one i.v. infusion for four weeks, consisting of  $10^7$ - $10^9$  cells/sq.m of body surface area, is used. Established protocols are described, for example, in Greenberg, P.D., Adv. Immunol. 49 (1991) 281-355, and Riddel, R.R., et al., Science 257 (1992) 238-241. At the time of adoptive transfer, patients can be vaccinated with

immunogenic tumor cells as well as treated with soluble recombinant tumor-specific B cell molecules to provide further re-stimulation in vivo. This can be achieved by injecting i.v. in the patients a predetermined amount of the soluble B7 conjugate, whereby the mAb binds to the surface of the cells of the tumor diagnosed. If the tumor expresses more than one suitable marker, more than one B7 x anti-tumor mAb molecule can be injected into the patient. The amount of soluble reagents and the schedule of injections are determined during the preclinical and clinical trials using, for instance, radio-labeled proteins to monitor its clearance and the efficiency of targeting into the tumor mass. All the essential parameters for this kind of treatment must be derived from the conventional experience of pharmacological and nuclear medicine and are not difficult to find out. It will also be essential to monitor the neutralizing antibody response that the patients can mount against the recombinant proteins.

As mentioned above, it is preferred to monitor the activated T cells in vivo after application. In this case, the adoptive immunotherapy is based on ex vivo expansion of tumor-specific T cells, which are marked preferably with LNGFR and reinnoculum into a patient, preferably together with the tumor-specific soluble B7 conjugate. In this case, the soluble B7 conjugate will re-stimulate transferred tumor-specific effector T cells at the site of the residual tumor mass, allowing the optimal amplification in vivo of the immune response. In fact, in the absence of soluble B7 conjugates (or in the absence of another adequate costimulation), the transferred antitumor T cell blast may perform fewer cycles of killing, after which they can be functionally inactivated or physically eliminated by programmed cell death. The marking of the tumor-specific T cells transferred during the adoptive immunotherapy may allow to monitor the efficiency of this approach by determining the persistence in the patient of transferred T cells.

The following examples and the drawing are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

**Fig. 1** describes the proliferation of human primary  $CD4^+CD45R0^+$  T cells costimulated by recombinant soluble B7-Ig molecules (A: anti-CD3; B: anti-CD3<sup>+</sup> B7-2IgG3; C: anti-CD3<sup>+</sup> B7-1IgG1).

**Fig. 2 A** describes the survival of tumor-specific T cell lines. Anti-tumor autologous CTL line can be induced, maintained and expanded in vitro only by B7-2 IgG3

targeted RMA-Thy1.1 cells, and not by control targeted CD4-IgG1 cells. (a: re-stimulation; b: adoptive transfer)

**Fig. 2 B** describes that a specific anti-RMA T cell line but not an anti-J558L T cell line efficiently cures mice inoculated with RMA lymphoma cells. The CTL lines, induced and propagated in vitro by RMA-Thy1.1 cells targeted with B7-2-IgG3 molecules, have 100% therapeutic activity when adoptively transferred into mice bearing an established RMA tumor. Furthermore, 80% of the cured mice develop a systemic immunity against a subsequent challenge with RMA cells. (a: challenge with wt RMA; mice are treated with: PBS / IL-2 only / CD8 anti-J558L + IL-2 / CD8 anti-RMA + IL-2).

**Fig. 3 A** describes the survival of mice inoculated with B7-2 IgG3 pre-targeted RMA Thy1.1 living cells. Primary rejection of 40% of mice challenged with living RMA-Thy1.1 cells, pre-targeted in vitro by three-step procedure with B7-2-IgG3.

**Fig. 3 B** Describes the therapeutic efficacy of B7-2 IgG3 targeted, non-replicating RMA Thy1.1 cells. Only non-replicating RMA-Thy1.1 cells, pre-pulsed in vitro by three-step with B7-2-IgG3 can cure 60% of mice bearing an established tumor.

#### **Example 1**

##### **Soluble B7-1IgG1 and B7-2IgG2 costimulate proliferation of primary CD4<sup>+</sup>CD45R0<sup>+</sup> human T cells**

Purified soluble recombinant B7-1Ig and B7-2Ig molecules (Traunecker, A., et al., Immunology Today 10 (1989) 29-31 and Traunecker, A., et al., Nature 331 (1988) 84-86) were tested for their ability to costimulate proliferation of highly pure human primary CD4<sup>+</sup>CD45R0<sup>+</sup> T lymphocytes. T cells were activated with suboptimal concentration of anti-CD3-specific antibody in the presence of increasing concentration of soluble B7-1Ig or B7-2Ig cross-linked to plastic well. As shown in Fig. 1, both the soluble B7 molecules costimulate in a dose response manner the proliferation of human primary T cells.

**Example 2****Soluble B7-1IgG1 costimulates the acquisition of cytotoxic activity in human primary CD8<sup>+</sup> T cells against allogeneic tumor target cells**

Primary human CD8<sup>+</sup> T cells were purified to homogeneity (purity of 90%) by negative depletion of CD4<sup>+</sup> T cells, using anti-CD4 mAb and magnetic beads. One million purified CD<sup>+</sup> T cells were restimulated in vitro with 5 millions of irradiated Jurkat lymphoma cells, in the presence of insoluble B7-1IgG1 molecules, B7-1IgG1 and IL-2, IL-2 alone or the control chimeric molecule CD4-IgG1. After 5 days, responding T cells were purified on a Ficoll gradient and tested for cytotoxicity against Jurkat cells at various effector to target ratios. Fig. 2 shows that CD8<sup>+</sup> T cells stimulated by Jurkat cells in the presence of both B7-1IgG1 and IL-2 kill the target more efficiently than IL-2 alone. Thus, soluble B7-1Ig molecules are able to induce the acquisition of effector function in CD8<sup>+</sup> T cells.

**Example 3****Biotinylation of antibodies**

Biotinylations are performed using a succinimide ester of biotin. The coupling is done through free amino groups on the antibody or other protein, normally lysyl residues. A solution of N-hydroxysuccinimide biotin at 10 mg/ml in dimethyl sulfoxide and an antibody solution of at least 1-3 mg/ml sodium borate buffer (0.1 M, pH 8.8) are prepared. If antibodies have been stored in sodium azide, the azide must be removed prior to coupling by dialyzing extensively against the borate buffer to remove. The biotin ester is added to the antibody at a ratio of 25-250 µg of ester per milligram of antibody, mixed well and incubated at room temperature of 4 hr.

High concentrations of the biotin ester will lead to multiple biotin groups binding to the antibody and will increase the probability that all of the antibodies will be labeled. Lower ratios will keep biotinylation to a minimum (25 µg of ester/mg of antibody gives an initial molar ratio of approximately 10:1). 20 µl of 1 M NH<sub>4</sub>Cl per 250 µg of ester is added and the mixture is incubated for 10 min at room temperature. The antibody solution is dialyzed against PBS or other desired buffer to remove uncoupled biotin. The biotin dialyzes more slowly than would be expected for its size, so extensive dialysis is needed.

**Example 4****Plasmocytoma cells secreting B7-1Ig or B7-2Ig molecules induce an efficient protective antitumor immunity in syngeneic mice**

J558L plasmocytoma clones secreting B7-1IgG1, B7-1IgM, B7-2IgG3, CD4IgG1, CD4IgM or CTLA4IgG1 were injected in the right flank of syngeneic Balb.c mice and their growth was evaluated. None of the mice challenged with plasmocytoma cells secreting B7-1 or B7-2 molecules developed a tumor mass, whereas tumors developed in all the mice challenged with cells secreting control CD4 or CTLA4 molecules. These results are summarized in Table 1.

Furthermore, all the mice that rejected J558L cells secreting B7 Ig molecules showed a protective secondary response to later challenge with parental plasmocytoma cells (Table 1). Thus, soluble chimeric B7-1 and B7-2 molecules maintain in vivo the capacity to prime an efficient antitumor immunity and to vaccinate animals against tumor onto which they are targeted.

**Table 1:** J558L plasmocytoma cells secreting human B7-1Ig or B7-2Ig can be used to vaccine syngeneic animals against later challenge with non-transfected parental cells.

Tumor type	% of tumor-free mice primary response	% of mice showing memory response
J558L	0	0
J558L-B7-1IgG1	100	100
J558L-B7-1IgM	100	100
J558L-B7-2IgG2	100	100
J558L-B7-CD4IgG1	0	0
J558L-B7-CD4IgM	0	0
CTLA4IgG1	0	0

**Example 5****Induction of autologous CTLs**

The gene coding for Thy1.1 allele was transfected into two mouse tumorigenic cell lines (T lymphoma RMA and mammary adenocarcinoma TS/A) that are syngeneic to mouse strains that express the Thy1.2 allele (C57.B6 and Balb.c respectively). In this way, once the tumor

cells are inoculated into the mice, they will form the only tissue of the mouse expressing the specific tumor marker. It is also preferred to use as the tumor marker the alleles Ly2.1 (CD8) and Ly5.1 (CD45) for improvement of behavior on the tumor cell surface (i.e. easy capping, internalization or sheeding). There are used mAbs specific for the desired allelic tumor marker, which will represent the tumor targeting arm. They are used as such, or they can be reduced to -46ab' fragment.

#### **Priming with RMA-Thy1.1+ (tri-step)**

Non-replicating (irradiated or mitomycin C-treated) RMA or TS/A cells expressing Thy1.1 are pulsed at +4°C with a saturating dose of biotinylated Thy1.1 specific monoclonal antibody (mab 19E12). The excess mab is washed out and an excess amount of Neutravidine (NAv, recombinant avidine from Pierce) is added. The NAv is tetravalent and binds to biotinylated 19E12 mab while still leaving at least one free binding site for another biotinylated molecule. The excess NAv is washed out and either the biotinylated recombinant B7-1IgG1 or the B7-2IgG3 are added, to complete the three-step bridge. As a control, priming with RMA cells transfected with Thy1.1 is used. After 6 days, the surviving T cells were assayed in a killer assay against parental RMA cells.

The tumor cells now express on their surface potent costimulatory molecules in the absence of any genetic manipulation.

The tri-step pulsed tumor cells are used to re-stimulate in vitro allogeneic cytolytic CD8<sup>+</sup> T cells.

In fact, only cells expressing functional B7-1 or B7-2 on their surface can prime or re-stimulate autologous effector cell in vitro. The results are shown in Table 2 and Fig. 2.

**Table 2:** The killing is specific, and there is no NK killing on NK-targets (YAK or RMA-S cells).

Priming with	E/T 100:1	50:1	25:1	12.5:1	6:1	3:1
RMA-Thy1.1+ tri-step	30%	15%	12%	8%	7%	5%
RMA-Thy1.1	5%	5%	5%	4%	3%	0%

E/T = Effector/Target Ratio

#### **Example 6**

##### **Induction of a protective response by tri-step pre-pulsed tumor cells**

Living tumor cells (e.g., RMA-Thy1.1) prepulsed in vitro with soluble B7-1 or B7-2 (e.g., B7-2-IgG3) using the tri-step procedure are inoculated subcutaneously (s.c.) into syngeneic mice and scored for rejection. Moreover, non-replicating tumor cells prepulsed in vitro with the tri-step are used to vaccinate mice from a second challenge with a lethal dose of parental cells (results see Fig. 3 A)

#### **Example 7**

##### **Induction of a curative immunity against minimal tumor residual disease**

Mice are challenged either s.c. or intravenously (i.v.) with various doses of living tumor cells expressing Thy1.1 allele (or other tumor-specific markers). The tumors are allowed to grow for some days, then they are treated with non-replicating RMA-Thy1.1 cells, pre-targeted in vivo by three-step with B7-2-IgG3. The non-replicating, therapeutic cell vaccine is given s.c. twice a week for three weeks (results see Fig. 3 B).

#### **Example 8**

##### **Induction of a curative immunity against minimal tumor residual disease, by direct in vivo three-step administration of B7-Ig molecules**

Mice are challenged either s.c. or intravenously (i.v.) with various doses of living tumor cells expressing Thy1.1 allele (or other tumor-specific markers). The tumors are allowed to grow for some days, then they are treated with the tri-step in vivo. First, they receive the

biotinylated anti-Thy1.1 mab; 12 hours later NAv, and 36 hours later the biotinylated soluble recombinant B7-1 B7-2. The tumor growth is then scored. The in vivo tri-step can be repeated over time and it can be associated with the use of recombinant, biotinylated lymphokines which can synergize with B7-1 or B7-2 (IL-4, IL-7, IL-10, IFN-gamma, IL-12) at the site of the tumor mass.

Induction of a curative response against minimal tumor residual diseases by combining in vivo tri-step targeting of soluble B7-1 or B7-2 costimulatory molecules with active vaccination with engineered tumor cells and/or passive transfer of tumor-specific T cell lines expanded in vitro with the in vivo re-stimulation.

The engineered tumor cells used as vaccines can be generated either by transfection with genes coding for B7-1 or B7-2 or by tri-step prepulsing procedure. On the same line, tumor-specific T cells can be induced and expanded in vitro following the tri-step protocol described above.

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**Patent Claims**

1. A method for the production of activated tumor-specific T cells by co-cultivating, ex vivo, tumor cells from a patient with T cells from that patient, comprising the steps of
  - i) incubating the tumor cells with a first fusion protein obtained from a B7 protein and one partner of a biological binding pair and a second fusion protein obtained from an antibody against a cell surface antigen and the other partner of the biological binding pair;
  - ii) inhibiting the proliferation of the tumor cells prior to or after that incubation;
  - iii) co-cultivating the tumor cells with the T cells to be activated, until activation of the T cells is attained;
  - iv) separating the activated T cells from the tumor cells.
2. The method according to claim 1, characterized in that the proliferation of the tumor cells is inhibited prior to or after the incubation according to step i).
3. The method according to claim 1 or 2, characterized in that the activated T cells are marked prior to or after the activation according to step iii).

Fig. 1

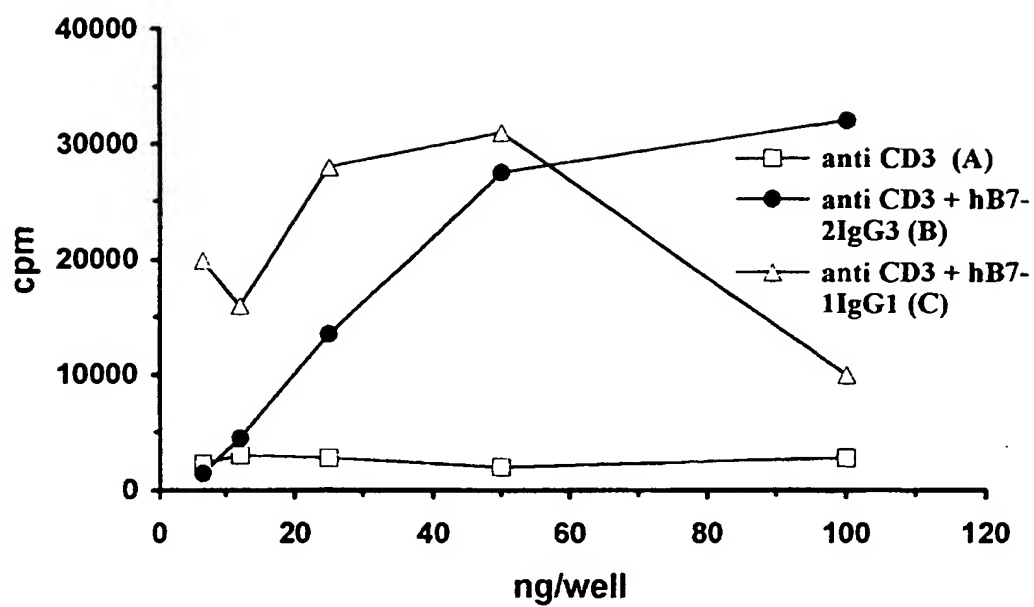


Fig. 2

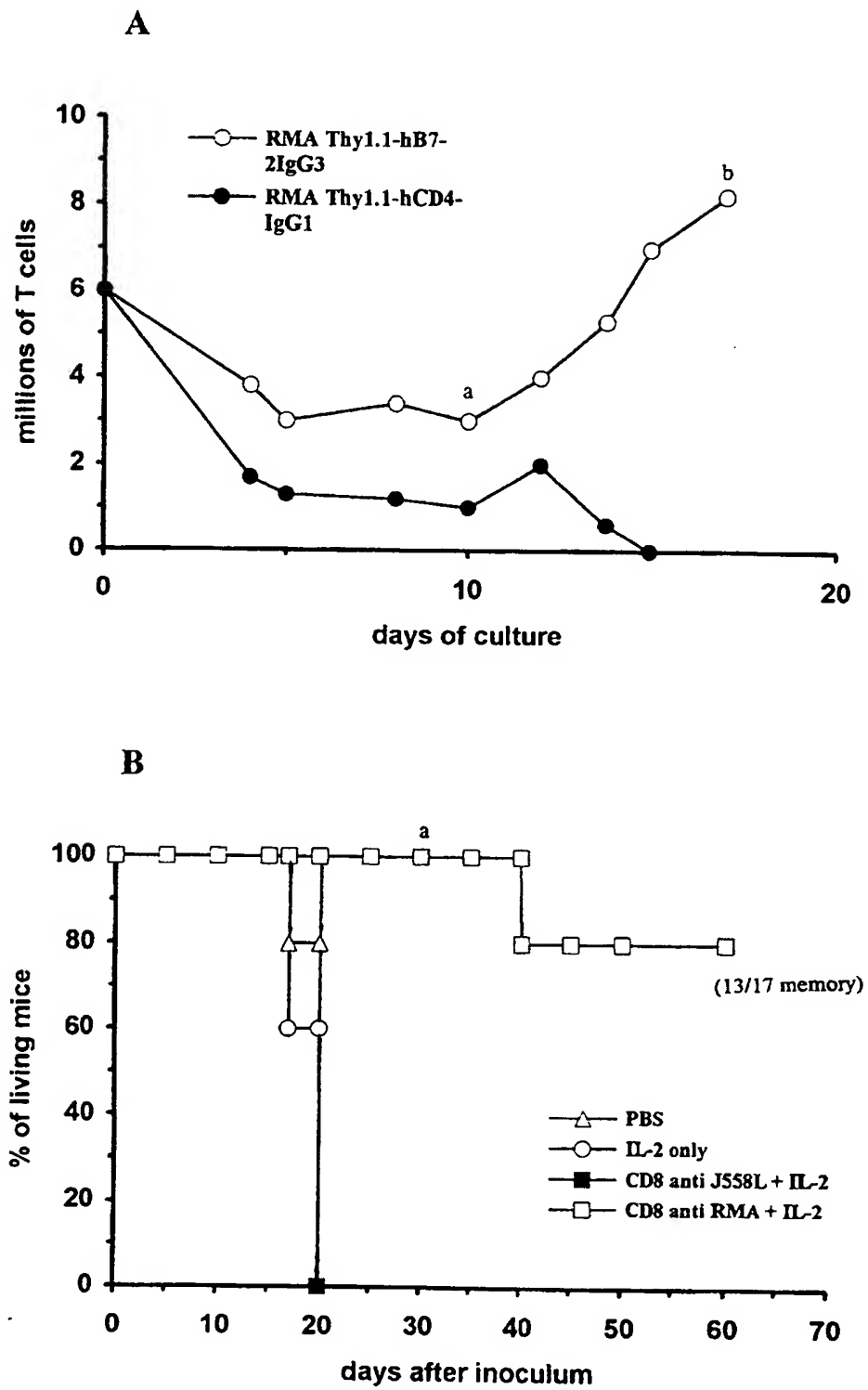
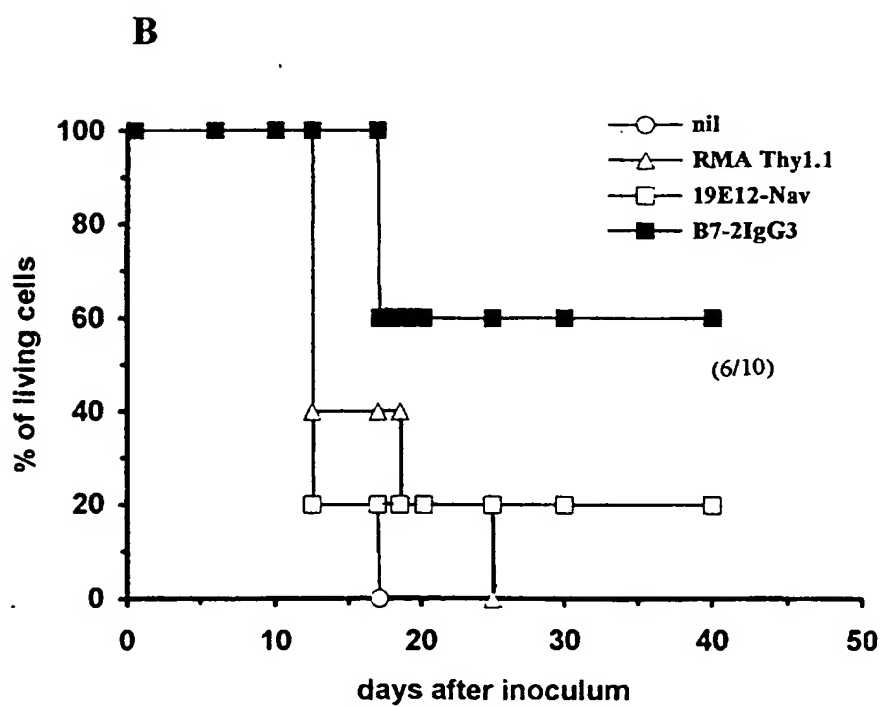
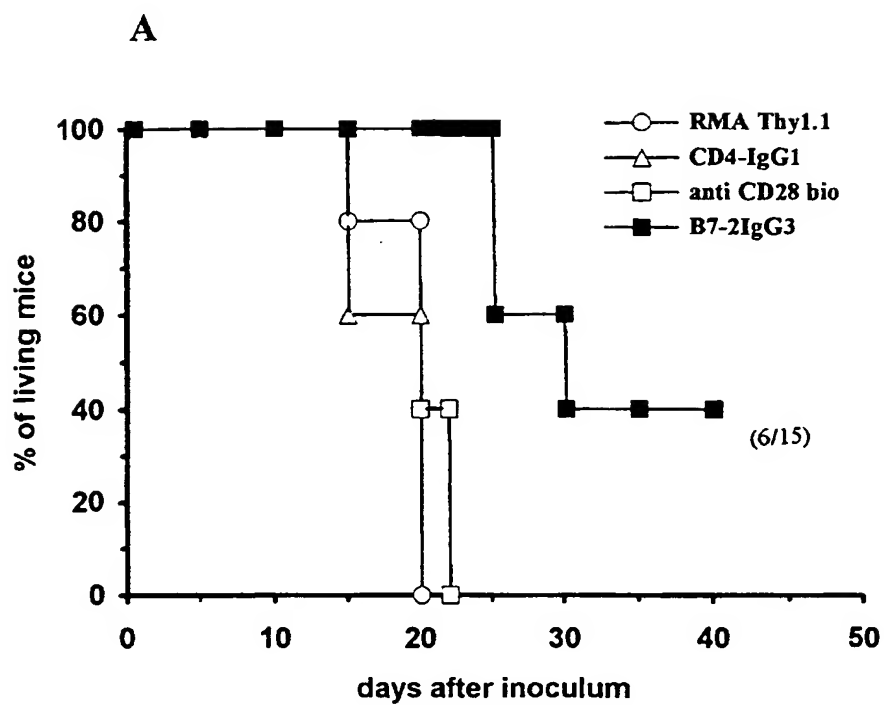


Fig. 3



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 97/01541

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 03408 A (DANA FARBER CANCER INST INC ;REPLIGEN CORP (US)) 2 February 1995 cited in the application see page 2, line 21 - line 37 see page 5, line 16 - line 29 see page 6, line 5 - page 7, line 13 see page 38, line 35 - page 40, line 21 see page 42, line 23 - page 43, line 3 ---	1-3
Y	EP 0 496 074 A (MINISTERO DELL UNI E DELLA RIC) 29 July 1992 see the whole document ---	1-3
Y	WO 87 04628 A (HUTCHINSON FRED CANCER RES) 13 August 1987 see page 4, line 5 - line 32 ---	1-3
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☒ Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search

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# INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>THE JOURNAL OF IMMUNOLOGY, vol. 149, no. 4, 15 August 1992, pages 1115-1123, XP002012843 AZUMA ET AL: "INVOLVEMENT OF CD28 IN MHC-UNRESTRICTED CYTOTOXICITY MEDIATED BY A HUMAN NATURAL KILLER LEUKEMIA CELL LINE" cited in the application * page 1115,abstract *</p> <p style="text-align: center;">-----</p>	

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information on patent family members

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